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(54) METHODS FOR PURIFYING NUCLEIC ACIDS

VERFAHREN ZUR REINIGUNG VON NUKLEINSÄUREN

PROCEDE DE PURIFICATION D'ACIDES NUCLEIQUES

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Description

Field of the Invention

[0001] The invention relates to methods for producing high purity nucleic acids. Specifically, the invention relates to producing pharmaceutical quality nucleic acids. The invention relates in particular to methods for preparing pharmaceutical quality plasmid DNA.

Background of the Invention

[0002] Since the advent of recombinant DNA, methods have developed and improved for the purification of DNA and RNA to further molecular biology research. While these methods have allowed considerable study of nucleic acids in research environments, they have not addressed issues involved in the human clinical use of purified nucleic acids such as is required for many current gene therapy protocols.

[0003] Gene therapy involves the introduction of nucleic acid into a patient's cells, which, when expressed, provide a therapeutic benefit to the patient. Examples include the introduction of an exogenous, functional gene to correct a genetic defect in a patient carrying a defective gene or to compensate for a gene that is not expressed at sufficient levels. Other examples include the introduction of mutant genes, antisense sequences or ribozymes to block a genetic function, e.g., in the treatment of viral infections or cancer.

[0004] Much of the focus in gene therapy has been on using viral vectors, especially retroviral vectors, for introducing exogenous nucleic acid into a patient's cells. To date, most of these protocols have been for *ex vivo* gene therapy, in which the patient's cells are first removed from the patient, genetically modified *ex vivo*, and then returned to the patient. The alternative to *ex vivo* gene therapy is *in vivo* gene therapy. *In vivo* gene therapy refers to the introduction of exogenous genetic capability directly to the patient where it is taken up by the target cells, which then express the introduced gene to produce a therapeutic product. Viral vectors have been used for *in vivo* gene therapy although their use is associated with a number of drawbacks, e.g. immunogenicity of the viral vector and safety concerns such as insertional mutagenesis or viral contamination.

[0005] Other means of in vivo gene delivery include the introduction of naked DNA into the target tissue of interest, or the use of lipid-mediated DNA delivery. Typically, introduction of naked DNA will be used when the exogenous genetic capability is to be introduced directly into the target tissue. By complexing with liposomes or lipids, DNA is compacted, allowing systemic delivery of the lipid/DNA complexes to various tissues of interest. See ACT patent application WO 93/25673. Lipid/DNA complexes can be targeted to particular tissues by altering the lipid composition, lipid/DNA ratio, mode of delivery, etc.

[0006] For any application in which nucleic acid is introduced into a patient, there is a need to produce highly purified, pharmaceutical grade nucleic acid. Such purified nucleic acid must meet drug quality standards of safety, potency and efficacy. In addition, it is desirable to have a scaleable process that can be used to produce large quantities of DNA, e.g., in the range of 100s of milligrams to 100s of grams. Thus, it is desirable to have a process for producing highly pure nucleic acid that does not use toxic chemicals, known mutagens, organic solvents, or other reagents that would compromise the safety or efficacy of the resulting nucleic acid, or make scale-up difficult or impractical. It is also desirable to prepare nucleic acids free from contaminating endotoxins, which if administered to a patient could elicit a toxic response. Removal of contaminating endotoxins is particularly important where the nucleic acid is purified from gram negative bacterial sources, e.g. plasmid or bacteriophage DNA, which have high levels of endotoxins.

[0007] The invention described below meets these needs and provides other related advantages as well.

Relevant Literature

[0008] Lis *et al.*, (1975) *Nucleic Acids Res.* 2: 383-389 describe polyethylene glycol-based DNA purification methods. Zasloff *et al.*, (1978) *Nucleic Acids Res.* 5:1139-1153 describe acid phenol purification of plasmid DNA. Sambrook *et al.*, (1989) Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, describe several different methods for relatively small scale, research grade methods of plasmid DNA purification. Ausubel *et al.*, eds. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York, also disclose different methods for relatively small scale methods for purifying plasmid DNA for research use.

[0009] Chandra *et al.*, (1992) *Anal. Biochem.* 203: 169-172 describe the use of Hi-Load Q Sepharose column chromatography for preparing plasmid DNA. Marquet *et al.*, (1995) *BioPharm* September 1995: 26-37 and Horn *et al.*, (1995) *Hum. Gene Therapy* 6: *565*-573 discuss process development issues and techniques including the use of polyethylene glycol and gel filtration/size exclusion chromatography to prepare plasmid DNA. Davis *et al.*, (1996) BioTechniques 21:92-99 compares cesium chloride and anion-exchange purified plasmid DNA for gene transfer efficiency. High performance liquid chromatography using anion-exchange chromatography resins to purify plasmid DNA are

described in Thompson (1986) *BioChromatography* 1(2): 68-80 and Coppella, *et al.*, (1987) *J. Chromatography* 402: 189-199.

[0010] The principles, theory and devices used for tangential flow filtration are described in Michaels, S. L. *et al.*, "Tangential Flow Filtration" in Separations Technology, Pharmaceutical and Biotechnology Applications, W. P. Olson, ed., Interpharm Press, Inc., Buffalo Grove, IL (1995). Procedures for removing endotoxin from biological samples are described in Sharma, (1986) *Biotech. and Applied Biochem.* 8: 5-22; Vanhaecke, *et al.*, (1989) *J. Clin. Microbial.* 27 (12): 2710-2712; Weiss, *et al.*, Sartorius Corporation, Developing Methods #7, "Endotoxin Removal," Edgewood, NY; Talmadge *et al.*, (1989) *J. Chromatography* 476: 175-185; and Hou *et al.*, (1990) *Biotech. and Applied Biochem.* 12: 315-324. Montbriand, et al., (1996) *J. Biotechnology* 44: 43-46, describe removal of endotoxin from DNA using polymyxin B resin.

SUMMARY OF THE INVENTION

[0011] The present invention is directed to a method for purifying a nucleic acid from a solution, comprising filtering the solution through an ultrafiltration unit composing a gel layer to provide a permeate solution and a retentate solution whereby the nucleic acid is retained in the retentate solution, and collecting the retentate solution to provide a purified nucleic acid solution.

[0012] Preferably the nucleic acid is DNA, particularly viral or plasmid DNA. The ultrafiltration unit is preferably an open-channel, flat plat device or a hollow fiber device. For must nucleic acids, the ultrafiltration membrane should have a molecular weight cut-off of between 1K and 1,000K, preferably around 50K to 500K for plasmid DNA, and most preferably around 300K to 500K. Higher purities are obtained when ultrafiltration is performed under conditions allowing a gel layer to form. The gel layer may be allowed to form for up to about 90 minutes, using pressure of from about 5 psi (34.5 kpascal) to about 30 psi (206.8 k pascal), preferably around 10 psi (68.9 kpascal) to 20 psi (137.9 kpascal). The nucleic acid solution may be concentrated in the range of about 2-fold to about 50-fold during the ultrafiltration step.

[0013] In further preferred embodiments, plasmid DNA is further purified after tangential flow ultrafiltration by filtering the retentate solution through a 0.2μm filter and applying the filtered plasmid DNA solution to a positively charged ion exchange chromatography resin wherein the plasmid DNA is eluted from the ion exchange chromatography resin with a saline gradient. The purified plasmid DNA may be further purified by an additional diafiltration step, and optionally, further concentrated and/or exchanged into a buffer suitable for the intended use of the purified plasmid DNA.

[0014] The invention further provides a pharmaceutical composition comprising the nucleic acid prepared according to the method of the invention. The pharmaceutical composition is preferably plasmid DNA and comprises less than about 100 endotoxin units per milligram nucleic acid, less than about 2% RNA, less than about 1% single stranded DNA, less than about 0.1% protein, less than about 1% genomic DNA and more than 90% closed circular plasmid DNA.

[0015] It is an advantage of the present invention that nucleic acid is prepared without the use of organic solvents, including phenol, chloroform, ether, ethanol, isopropanol, isoamyl alcohol, n-butanol or other organic solvents. The use of such solvents pose safety and regulatory concerns due to the possibility of trace amounts in the final product. In addition, such solvents are toxic and inflammable, and pose serious risk and disposal problems if used in the quantities required for large scale purification. The purified nucleic acid of the present invention is also substantially free of contaminating endotoxin.

[0016] It is also an advantage of the present invention that nucleic acid prepared according to the inventive method is highly purified. Highly purified nucleic acid is advantageous for safety reasons as well as for improving reproducibility and efficacy of processes using such nucleic acid. In particular, highly purified DNA of the present invention is advantageously used for gene delivery using lipid carriers, whereby reproducible, high transfection efficiencies are obtained.

[0017] Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] Figure 1 is a schematic diagram of a tangential flow ultrafiltration process.

[0019] Figure 2 illustrates a schematic diagram of large-scale plasmid DNA purification as described in Example 1.

[0020] Figure 3 is a schematic representation of the plasmid p4119.

[0021] Figure 4 is a graph of HPLC analyses of a p4119 preparation before TFU (Panel A) and the retentate fraction obtained after TFU (Panel B) as described in Example 1.

[0022] Figure 5 shows the results of agarose gel electrophoresis of p4119 final purification product as described in Example 1. From left to right, the lanes are: (a) molecular weight markers, (b) p4119 cut four times, (c) p4119 cut two times, (d) p4119 cut one time, and (e) uncut p4119.

[0023] Figure 6 is a graph of HPLC analysis of the final product of the p4119 purification described in Example 1.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions:

[0024] "Diafiltration" is a mode of operating an ultrafiltration system in which the retentate is continuously recycled and diluted with fresh wash solution to replace that removed as permeate. Diafiltration will generally provide a cleaner separation of macromolecules retained in the retentate sample while the smaller molecules pass through into the filtrate. It may also be used to perform solvent removal or buffer exchange in the same step. "Continuous diafiltration" refers to the continuous addition of fresh wash buffer as filtration takes place. "Discontinuous diafiltration" refers to the repeated steps of concentrating the sample by ultrafiltration, and rediluting with buffer.

[0025] A "gel-layer," refers to a thin gelatinous layer of biomolecules that can form on or in an ultrafiltration membrane. The gel layer is generally a cohesive, adherent layer of constant solute concentration, also commonly referred to as concentration polarization. It usually will have some degree of hydraulic permeability depending on the nature of the solute forming the layer. "Gel-layer controlled" ultrafiltration refers to filtration conditions where the gel layer becomes the limiting factor to filtrate flow rate, and further pressure increases have little or no effect. By contrast, "membrane controlled" conditions are those in which the filtrate flow rate is controlled by the permeability of the membrane and the applied pressure.

[0026] An "open channel" filter is one which does not have a screen in the feed channel. By contrast, a "screen channel" or "closed channel" is a filter that has a screen in the feed channel.

[0027] "Permeate" refers to that portion of a sample that passes through the ulirafiltration membrane, and is also termed the "filtrate."

[0028] "Retentate" refers to that portion of a sample that does not pass through the ultrafiltration membrane.

[0029] "Tangential flow" or "cross-flow" filtration refers to a filtration process in which the sample solution circulates across the top of the membrane, while applied pressure causes solute and small molecules to pass through the membrane.

[0030] "Ultrafiltration" refers to a technique to separate particles by filtration through membranes having pore sizes ranging from about 0.001μm to about 0.05μm. Ultrafiltration membranes typically have a molecular weight cut-off (MWCO) in the range of 1,000 to 1,000,000 daltons. The MWCO typically is defined as the molecular weight of the globular solute which is 90% retained by that membrane. See Filtron Catalog, 1995/96, p.5. The actual molecular weight of particles that pass through or are retained by a membrane will depend on the size as well as the conformation and charge of a given molecule, the nature or the membrane pore or matrix, and the conductivity and pH of the solution.

[0031] It has now been found that nucleic acids can be highly purified from a mixture of substances, including proteins, cell debris, endotoxin, small degraded nucleotides, and the like, by tangential flow ultrafiltration (TFU). This technique is simple, efficient and yields very high purity nucleic acid in a single step, often on the order of 95% to 100% pure by HPLC analysis. It is also conveniently combined with diafiltration in a single step whereby the nucleic acid solution may be concentrated and/or exchanged into a different buffer solution to remove solvents, salts, and the like.

[0032] Nucleic acids that may be purified and/or concentrated according the methods described herein include DNA, RNA and chimeric DNA/RNA molecules, and may be from any biological source including eukaryotic and prokaryotic cells, or may be synthetic. Nucleic acids that may be purified include chromosomal DNA fragments, ribosomal RNA, mRNA, snRNAs, tRNA, plasmid DNA, viral RNA or DNA, synthetic oligonucleotides, ribozymes, and the like. Preferred are viral nucleic acids, and extrachromosomal DNAs. Of particular interest are plasmid DNAs encoding therapeutic genes. By "therapeutic genes" is intended to include functional genes or gene fragments which can be expressed in a suitable host cell to complement a defective or under-expressed gene in the host cell, as well as genes or gene fragments that, when expressed, inhibit or suppress the function of a gene in the host cell including, e.g., antisense sequences, ribozymes, transdominant inhibitors, and the like.

[0033] Thus, e.g., viral DNA or RNA may be purified from prokaryotic or eukaryotic viruses, in which the viral particles are initially purified from cultures or cells permissive for viral infection in accordance with conventional techniques, e.g., from bacterial, insect, yeast, plant or mammalian cell cultures. Extrachromosomal DNAs include autonomously replicating DNAs from a variety of sources including, e.g., mammalian cells (*see, e.g.*, Yates et al., *Nature* (1985) 313: 812-815; Heinzel et al., *Mol. Cell. Biol.* (1991) 11(4): 2263-2272), plant cells, yeast cells (e.g., 2μm plasmids), and prokaryotic cells. Plasmid DNA isolated from prokaryotic cells include naturally occurring plasmids as well as recombinant plasmids encoding a gene of interest including, e.g., marker genes or therapeutic genes.

[0034] Initial preparatory purification of the nucleic acid sample before tangential flow ultrafiltration will depend on the source of the nucleic acid and the level of purity desired. Ideally, many of contaminants are removed by one or more coarse purification steps before tangential flow ultrafiltration to reduce the number of contaminating particles that could foul the ultrafiltration membrane, impeding performance, and decrease the amount of any larger contaminants that would be retained with the nucleic acid. For nucleic acids obtained from biological sources, e.g. tissues and cells, including cell lines, mammalian, yeast, plant or bacterial cells, initial preparatory steps to lyse cells and remove cell

components, e.g. proteins, cell walls or membranes, can be performed using conventional methods known to those of ordinary skill in the art. *See, e.g.*, Sambrook et al., 1989; Ausubel et al., 1989. For purification of extrachromosomal DNA, such as plasmid DNA, it is desirable to use methods that do not shear chromosomal DNA, making its removal simpler and avoiding contamination with the final plasmid DNA product. Thus, for example, plasmid DNA may be isolated from bacterial sources using conventional procedures including lysis with alkali and/or detergents, e.g. SDS, NP40, Tween 20, etc., followed by precipitation of proteins, chromosomal DNA and cell debris. For purification of extrachromosomal DNA from mammalian cells, e.g., a conventional Hirt extraction may be used. Hirt, B., (1967) *J. Mol. Biol.* 26:365-369. For synthetic nucleic acids, little or no pretreatment may be necessary before TFU.

[0035] If pharmaceutical grade nucleic acid is desired, it is highly preferred that the preparatory steps do not include the use of organic solvents or toxic chemicals that may raise safety and regulatory concerns. Example 1, below, exemplifies a method for producing high purity pharmaceutical grade plasmid DNA, without use of organic or toxic chemicals, using the methods of the present invention.

[0036] Figure 1 is a schematic diagram of a tangential flow ultrafiltration process. Briefly, the feed tank 10 comprises the sample solution to be filtered. The solution enters the filtration unit 50 through the feed channel or feed line 20. The circulation pump 30, located in the feed line 20 controls the solution flow. The filtration unit 50 comprises the ultrafiltration membrane. Filtration through the ultrafiltration membrane separates the sample solution into a permeate solution and a retentate solution. The permeate solution exits the unit through the permeate channel or permeate line 60. Flow through the permeate channel may be controlled through a permeate valve located in the permeate channel 60. The retentate solution passes into the retentate channel or retentate line 90, which is circulated back into the feed tank 10. Pressure across the ultrafiltration membrane (transmembrane pressure or TMP) is measured by pressure detectors in the feed channel 40 and in the retentate channel 80. TMP is controlled by adjusting the retentate valve 100. When TFU is performed in diafiltration mode, diafiltration buffer 110 is added to the sample solution in the feed tank 10. When TFU is used to concentrate the sample solution, however, diafiltration buffer 110 is not added to the feed tank 10. System control can be manual or automated, with pressure transducers, flow meters, in-line conductivity meters, and other feedback loops.

[0037] The ultrafiltration membrane will be selected based on the size and conformation of the nucleic acid to be purified, and typically will have a molecular weight cut-off (MWCO) in the range of 1K to 1,000K daltons. For many supercoiled plasmid DNAs, ultrafiltration membranes having a MWCO around 300K to 500K daltons may be used. For some larger plasmids, however, improved speed, purity and quality of the resultant DNA is obtained when larger MWCO membranes are used. Preferably, therefore, plasmid DNA with sizes ranging from about 2 Kb to 15 Kb are purified using ultrafiltration membranes having a MWCO of 300K daltons; plasmids ranging from about 15 Kb to about 50 Kb may be purified using membranes having a MWCO of 500K daltons; and plasmids of about 40 Kb or larger may be purified using membranes having a *MWCO* of 1,000K daltons. With some hollow fiber ultrafiltration devices, e.g., those with symmetric pores, larger nominal pore sizes may be used. For example, plasmid DNA of up to about 5Kb can be purified using membranes having up to 500K daltons MWCO in a hollow fiber device.

[0038] Under these conditions, plasmid DNA will be retained in the retentate while contaminating substances including many proteins, cell membrane debris, carbohydrates, small degraded nucleotides, etc., pass through the membrane into the filtrate. Smaller nucleic acids, e.g., small synthetic oligonucleotides, may be purified using ultrafiltration membranes with a MWCO of around 1K to 5K daltons. For any nucleic acid to be purified, the optimal membrane pore size may be determined empirically using small scale devices, e.g., centrifugation devices, stirred cell devices, or small scale hollow fiber systems, available from a variety of commercial manufacturers. A manifold system may be used for optimizing parameters in process scale development. Commercial sources for ultrafiltration devices include Pall-Filtron (Northborough, MA), Millipore (Bedford, MA), and Amicon (Danvers, MA).

[0039] Many types of ultrafiltration devices useful in the present invention are commercially available including e.g., a flat plate device, spiral wound cartridge, hollow fiber, tubular or single sheet device. See Michaels *et al.*, (1995). Preferably, the ultrafiltration unit is a flat plate device or hollow fiber device.

[0040] It has been found that shearing of the nucleic acid is minimized if the filtration device used for TFU is an open-channel device. Screened channels inhibit formation of a gel layer and have been found to shear and decrease yield of the retained nucleic acid. Screen channels may be designed, however, having minimal compression of the screens such that the shearing and nucleic acid loss may be minimized.

[0041] The surface area of the ultrafiltration membrane used will depend on the amount of nucleic acid to be purified. Generally, about ten square feet of membrane is used per gram of nucleic acid. Thus, about five square feet of membrane is used per 200 to 800 mg nucleic acid; more typically, about five square feet of membrane is used for 400 to about 600 mg nucleic acid.

[0042] The membrane may be of a low-binding material to minimize adsorptive losses, and should be durable, cleanable and chemically compatible with the buffers to be used. A number of suitable membranes are commercially available, including e.g., cellulose acetate, polysulfone, polyethersulfone and polyvinylidene difluoride. Preferably, the membrane material is polyethersulfone.

[0043] It has been found that higher yields and purities are obtained when a gel-layer is allowed to form at the membrane surface before starting TFU. Thus, the sample solution initially is circulated through the ultrafiltration device 50, with the permeate valve 70 open and the permeate solution recirculated into the feed tank 10, for sufficient time to allow a gel-layer to form. The amount of time necessary for gel-layer formation may be determined empirically by monitoring the permeate solution for product loss, e.g., by HPLC analysis. The gel-layer is adequate once the product loss into the permeate is sufficiently low. Under preferred conditions, the gel layer acts as a second membrane barrier, which may cause nucleic acid molecules which would normally pass through the membrane to be retained. However, it is not necessary to perform the ultrafiltration under conditions, e.g., pressure and feed, that are fully gel-layer controlled. Thus, as used herein, filtration in the presence of a gel layer means that there is sufficient gel layer to cause additional solute retention beyond that resulting solely from the ultrafiltration membrane.

[0044] Typically, the gel layer is allowed to form for approximately 5 to 90 minutes, preferably around 20 to 60 minutes, depending on the device and size of the nucleic acid. For example, using a flat plate device to purify small plasmid DNAs (e.g., 2 Kb), the gel layer will be formed in about 60 to 90 minutes; for larger plasmid DNAs (e.g., 2-7 Kb), the gel layer will be formed in about 30 minutes. Using a hollow fiber device, the gel layer can be formed in approximately five to 30 minutes for most plasmid DNAs, or up to 45 minutes for plasmid DNAs less than about 2 Kb. After formation of the gel-layer, the permeate line 60 will be emptied into a waste receptacle and filtration allowed to proceed.

[0045] If a gel-layer is not allowed to form during an initial circulation period, product will usually be lost by leakage into the permeate solution. The amount of product loss will depend on the type of device used, the membrane MWCO and the total amount of nucleic acid in the sample. Thus in circumstances where product yield is not critical, the filtration may be performed without an initial circulation period while the gel layer forms. In such cases , the gel layer may be allowed to form during the initial period of filtration, after which product leakage into the permeate solution will decrease and product will then be retained in the retentate solution. For example, since a gel layer can be formed in a hollow fiber device in a short time period, (e.g., about five minutes for plasmid DNA of about 5 Kb, and about 150 mg DNA/sq. ft.), product loss during the initial period of forming the gel layer may not be significant and, therefore, re-circulation of the permeate solution into the feed tank while the gel layer forms may not be necessary.

[0046] Filtration will be performed using tangential flow to circulate the sample buffer as it crosses the membrane surface. During tangential flow filtration, pressure is applied across the membrane, which will allow smaller molecules to pass through the membrane while the retentate is recirculated. Typically, the flow rate will be adjusted to maintain a constant transmembrane pressure. Flow rate and pressure will usually fluctuate initially due to the formation of a gel layer. Generally, filtration will proceed faster with higher pressure and higher flow rates, but higher flow rates are likely to cause shearing of the nucleic acid or loss due to passage through the membrane. In addition, various TFU devices may have certain pressure limitations on their operation. The pressure, therefore, may be adjusted according to the manufacturer's specification. For flat plate devices, the pressure is preferably from about 5 psi (34.5 kpascal) to about 30 psi (206.8 kpascal), most preferably in the range of 10 psi (68.9 kpascal) to 20 psi (137.9 kpascal). For most plasmid DNAs, 15 psi (103.4 kpascal) to 20 psi (137.9 kpascal) is preferred. The circulation pump 30, will be selected to ensure minimal shearing of the nucleic acid. Typically, the circulation pump 30, is a peristaltic pump or diaphragm pump in the feed channel 20, and the pressure is controlled by adjusting the retentate valve 100.

[0047] Filtration will generally be performed in diafiltration mode. Optionally, the sample solution may initially be filtered without buffer addition until concentrated to a desired volume. Once concentrated, diafiltration buffer 110 is added and filtration continues to wash the retentate solution of contaminating small molecules and remove unwanted solvents and salts. Diafiltration may be either continuous or discontinuous. Preferably, diafiltration is continuous, and performed until from about 5 to about 500 volume equivalents have been exchanged, preferably about 10 to 100 volume equivalents. Generally, more diafiltration will be performed with increased contaminants bound to the nucleic acids, depending upon the purity required.

[0048] To further improve yield of the purified nucleic acid following TFU, the retentate solution is recirculated through the filtration unit 50, with the permeate valve 70 closed, for several minutes to remove residual nucleic acid. The retentate solution is collected, and additional diafiltration buffer 110 is added to wash the membrane filter. Typically, one to two volume equivalents of diafiltration buffer 110 will be used to wash the membrane filter. The retentate is again collected and combined with the original retentate solution containing the purified nucleic acid.

[0049] Nucleic acids purified by tangential flow ultrafiltration may be used directly or may be further purified depending on the level and type of contamination in the starting sample and the desired use. Typically, the nucleic acid purified by tangential flow filtration will be greater than 90% pure, often 95% to 100% pure as analyzed by HPLC. The nucleic acid thus purified may be used for a number of applications, e.g., molecular biological applications such as cloning or gene expression, or for diagnostic applications using, e.g., PCR, RT-PCR, dendromer formation, etc.

[0050] For therapeutic uses, e.g. use in gene therapy, it may be desirable to further purify the nucleic acid obtained from the tangential flow filtration step. In a preferred embodiment of the invention, the nucleic acid sample obtained from the tangential flow filtration step is subsequently filtered through a 0.2μm filter, further purified using ion exchange chromatography, and, optionally, filtered again through a 0.2μm filter. Desirably, the nucleic acid is further concentrated

and diafiltered using ultrafiltration, and filtered again through a 0.2μm filter as a final sterilization step.

[0051] Filtration through 0.2μm filters can be used to remove endotoxin and microorganisms while resulting in minimal nucleic acid loss. 0.2μm filters are available from a variety of commercial sources including, e.g., Pall-Filtron (East Hills, NY), Sartorius (Edgewood, NY), and Gelman (Ann Arbor, MI). Ideally, the filter used will be one that binds endotoxin while allowing nucleic acid to pass through. Pall Ultipor® N_{66}® filters have been found to remove substantial endotoxin with high yield of nucleic acid. Preferably, the nucleic acid solution is pre-filtered through a 0.45μm filter before filtration through the 0.2μm filter. Filters made for the removal of endotoxin, e.g., ion exchange filters, in many cases are not suitable for use with nucleic acid purification because the nucleic acid will bind to the filter.

[0052] Ion exchange chromatography may be used to further purify the nucleic acid, particularly from contaminating endotoxin, trace proteins, and residual cellular contaminants. A chromatography column is packed with an anion exchange chromatography resin. The optimal capacity of the column will be determined empirically based on the resin used and the size of nucleic acid to be purified.

[0053] Ion exchange chromatography resins are commercially available, including from EM Separations (Gibbstown, NJ), BioSepra (Marlborough, MA), Polymer Laboratories (Amherst, MA), Perseptive Biosystems (Cambridge, MA), Taso Hass (Montgomeryville, PA) and Pharmacia (Uppsala, Sweden). For most plasmid DNAs, preferred resins are those with no pore or with a large pore size, e.g., greater than 1000Å, preferably around 3000Å to 4000Å; with a medium bead size, e.g., about 20 to 500μm diameter; that do not leach matrix components. Ideally, the resin is also washable, e.g., with sodium hydroxide to allow repeated use.

[0054] A chromatography column is packed with an anion exchange chromatography resin. The optimal capacity of the column is determined empirically based on the resin used and the size of the nucleic acid to be purified. The column is packed under low pressure, typically less than about 7 bar. The pressure will depend on the resin used, and will usually be according to the manufacturer's specifications. Column pressure may be lower where resin pore size is smaller to limit trapping of the nucleic acid in the resin pores. Thus, for resins without pores, column pressure may be increased. The column is packed at about twice the anticipated flow rate in accordance with conventional techniques.

[0055] The nucleic acid sample is loaded onto the column in a loading buffer comprising a salt concentration below the concentration at which the nucleic acid would elute from the column. Typically, the salt concentration will be about 10 to 50mS, depending on the resin used. For weaker anion-exchange resins, a lower conductivity solution will be used, whereas for stronger anion-exchange resins, a higher conductivity solution will be used. The column will then be washed with several column volumes of buffer to remove those substances that bind weakly to the resin. Fractions are then eluted from the column using a shallow continuous saline gradient according to conventional methods, e.g., using up to 1.5M NaCl in a Tris-HCl buffer. Sample fractions are collected from the column. For intermediate scale preparations (e.g., from about 100 mg to about 3 grams nucleic acid), fractions will typically be at least 50ml to 2 liters where the nucleic acid peak is expected, then increased in volume past the expected peak. Analytical determinations of nucleic acid yield and purity are performed on each fraction. In addition, *Limulus* ameobocyte lysate (LAL) analyses may be performed on each fraction to determine residual endotoxin levels in each fraction. Fractions containing high levels of nucleic acid and low endotoxin are pooled. The resulting nucleic acid sample may again be filtered through a 0.2μm filter depending on the endotoxin levels and the desired purity.

[0056] For many applications it will be desirable to further purify the nucleic acid, lower the salt concentration of the resulting nucleic acid sample, concentrate the sample, and/or exchange the buffer to a more suitable buffer for subsequent uses. A final diafiltration step may be performed at this stage to achieve that result. If desired, a smaller MWCO ultrafiltration membrane may be used for this subsequent diafiltration step than used previously for purification, since the nucleic acid will be highly purified at this stage and predominantly small solute molecules will be passed through the membrane into the filtrate. For many plasmid DNAs, a 10K to 100K MWCO membrane may be used. Hollow fiber devices with about a 100K MWCO membrane are preferred at this stage, particularly when handling concentrated nucleic acid solutions, due to smaller hold-up volumes, increased flux, higher yields and shorter processing times.

[0057] Where DNA purified according to the above protocol is to be complexed with a lipid carrier for use in gene therapy, it is desirable to exchange the DNA into a low conductivity buffer, preferably by diafiltration. A low-conductivity buffer is meant to include any buffer of less than about 10 mS, preferably less than about 1 mS.

[0058] At a variety of places in the above protocol, analytical determination of nucleic acid yield and purity are advantageously performed. Typically, such assays are performed before and after each purification step, as well as to each nucleic acid-containing fraction from, e.g., preparative ion exchange chromatography. Preferred means for performing these analytical determinations include HPLC analysis of purity, spectrophotometric estimation of yield, silver staining and SDS-PAGE for protein analysis, and agarose gel electrophoresis and Southern blotting for DNA analysis.

[0059] The following Example illustrates certain aspects of the above-described method and advantageous results. The following example is shown by way of illustration and not by way of limitation.

EXAMPLE 1

Preparation of p4119 DNA

[0060] Pharmaceutical quality DNA was prepared as follows, using aseptic culture conditions for all cell culture procedures. Figure 2 is a schematic representation of the procedural steps.

[0061] An inoculum of *E. coli* containing plasmid p4119 (Figure 3) was prepared from frozen stock by the addition of 1ml of frozen (-80°C) bacterial culture to a 500ml foam-plugged flasks containing 100ml TB broth (Sambrook *et al.*, 1989) supplemented with carbenicillin (100μg/ml). Cultures were incubated at 37°C and shaken at 220rpm for approximately 6 hours. Culture growth was determined by visual inspection or by determining OD_{600}, whereby OD values between 0.5 and 5 were deemed acceptable.

[0062] 5ml of this culture was used to inoculate each of 4 bioreactors containing 10L TB media supplemented with carbenicillin (100μg/ml) and with 1 ml/10L Mazu DF204 antifoaming agent. These cultures were incubated at 37°C and stirred initially at about 300 rpm. The cultures were aerated and dissolved oxygen was controlled via cascade control loops, agitation, airflow, and oxygen enrichment to an average of about 40% saturation. Cultures were incubated for about 10 to 16h. After incubation, cell content of each culture was determined by OD_{600}; OD_{600} values ranged from 16 to 18. Cells were harvested by centrifugation in a refrigerated Carr continuous flow centrifuge.

[0063] The cell pellets were spread into thin sheets and frozen at -80°C until used for further plasmid purification. 3.2Kg of the cell pellet was resuspended in 16L Solution I (25mM Tris-HCl, pH 8, 10mM EDTA, 50mM dextrose) at room temperature with stirring at 150 rpm for 1h. RNase digestion was achieved by the addition of RNase (305mg RNase/Kg cell paste) and incubating the solution on ice for 2hrs. Cells were lysed by the addition of the cells to 32L Solution II (0.2 NaOH/1%SDS) in an ice bath. The solution is stirred using a Bow-Tie Stirrer (Cole Parmer, Vernon Hills, IL) for 25 min. This solution was then neutralized and cell debris and chromosomal DNA were precipitated by the addition of 16L ice-cold Solution III (3M potassium, 5M acetate, pH 5.5). The solution was mixed with a Bow-Tie Stirrer on ice for 25 min.

[0064] The precipitated material was removed form the neutralized cell lysis solution by centrifugation. The solution was aliquoted into 1L centrifuge bottles and cetrifuged at 5300rpm for 20 min at 2°C. The supematants were then decanted through two layers Miracloth (CalBiochem, La Jolla, CA) arranged at 90° to each other, into a container at room temperature. The decanted supernatants were then filtered through 1.2 and 0.2μm filters arranged in series. As an alternative to centrifugation at this stage, precipitated material may be removed by filtration through a diatomaceous earth material such as Celite® HYFLO SUPER CELL (Celite Corp., Lompoc, Calif.). See U.S. Patent No. 5,576,196.

[0065] Filtered materials were then pumped into an ultrafiltration unit and the DNA solution filtered by tangential flow filtration through a Pall-Filtron Omega open channel Centrasette unit using 25 ft^2 (2.3 x 10^4 cm^2) of polyethersulfone (PES) membrane having a MWCO of 300K. The solution was introduced into the unit under a pressure of 10 psi (68.9 kpascal), with the permeate channel open, and the solution allowed to circulate through the unit for about 50 min. until a gel layer was formed. The permeate channel was then directed to a waste receptacle, and the DNA solution was filtered at a pressure of 10 psi (68.9 kpascal) until the solution was concentrated to volume of about 3.6L. Diafiltration buffer (Tris-HCl, pH 8.5) was then added and the solution was continuously diafiltered at a pressure of 10 psi (68.9 kpascal), flow rate of about 1L/min, until approximately 50 volume exchanges were performed.

[0066] After diafiltration, the retentate was recirculated through the ultrafilter for 10 min with the permeate valve closed. The retentate was removed and the membrane washed twice by an additional 1L diafiltration buffer per wash for 10 min each, with the permeate valve closed. The wash solutions were added to the retentate and analyzed by HPLC and $OD_{260/280}$ analysis.

[0067] HPLC analyses were performed on a 4.6mm x 3.5cm HPLC column packed with TSK-GEL DEAE-NPR resin at a buffer flow rate of 1ml/min and monitored at 254 nm. Samples were diluted 1:20 with Buffer A (20mM Tris-HCl, pH 8) and injected onto the column in a volume of 25μl. Sample was eluted in a gradient of 0% Buffer B (20mM Tris-HCl, pH 8/ 2M KCl): 100% Buffer A to 60% Buffer B: 40% Buffer A over 9 min. HPLC analysis of the permeate (Panel A) and retentate containing plasmid DNA product (Panel B) are shown in Figure 4. Plasmid DNA is typically 95% pure, and often 100% pure as determined by HPLC at this stage.

[0068] Spectrophotometric analysis was performed at wavelengths of 250, 260, and 280 nm. Typical ratios for purified DNA are $OD_{260}/OD_{250} > 1.1$, and $OD_{260}/OD_{280} > 1.9$. A total of 2.307g of plasmid DNA was isolated and purified in the above procedure, having OD_{260}/OD_{250} of 1.1047 and OD_{260}/OD_{280} of 1.9290.

[0069] The recovered plasmid DNA was filtered twice through a Gelman Ground Water Capsule 0.45μm filter, followed by two filtrations through a Pall-Filtron Capsule N_{66} 0.22μm filter.

[0070] Plasmid DNA was further purified by ion exchange chromatography. DNA was loaded in a total volume of 4740 ml onto an Amicon Vantage A column packed with a 2.5L bed volume of TMAE-650 (M) (trimethylamino ethyl) Fractogel (EM Separations, Gibbstown, NJ). The column was equilibrated with Equilibration Buffer (50mM Tris, pH 8.5) at a LFV (linear flow velocity) between 80-150 cm/hr at 0.7 bar column pressure. The DNA was loaded at 225 ml/

min flow rate at 0.7 bar column pressure. The column was washed with 3 to 5 column volumes of Equilibration Buffer at 32-35 mS. DNA was eluted from the column over an elution gradient of from 32 mS to 59 mS or from 0.5M NaCl to 1.5M NaCl in 50mM Tris, pH 8.5, at a flow rate of 225 ml/min and a column pressure of 0.55 bar. Fractions were collected in volumes of 130 to 1650ml starting when the A_{260} was greater than 0.2 and ending when the A_{260} was less than 0.2.

[0071] All fractions were analyzed by HPLC and LAL endotoxin assay. The results are shown in Table 1. A total of 2044 mg DNA was loaded onto the column and 1946 mg were recovered in a total volume of 6079ml, a yield of 95.22%. Column fractions 2-10 were pooled (1905mg DNA and 3.73 x 10^5 EU LPS in 5941 ml). Fractions to be pooled were chosen to provide the maximum yield of recovered DNA while minimizing the amount of contaminating lipopolysaccharide (LPS) in the preparation.

TABLE 1

Fraction	DNA conc. (mg/ml)	Volume (ml)	Yield (mg)	LPS (EU/ml)	LPS/DNA (EU/mg)
1	0.30042	137.59	41.33	263.7	878
2	1.04234	531.01	553.49	353.2	339
3	0.88851	501.18	445.30	103.2	116
4	0.62513	481.00	300.69	53.6	85.7
5	0.43912	529.82	232.65	38.8	88.4
6	0.26979	585.56	157.98	35.0	130
7	0.14750	526.13	77.60	15.3	104
8	0.08712	568.91	49.56	11.8	135
9	0.05810	603.83	35.08	9.12	157
10	0.03288	1613.78	53.06	6.49	197

[0072] The recovered DNA solution was filtered through a Pall Ultipor N_{66} 0.2µm filter. To reduce the salt content, the DNA solution was subjected to a final diafiltration step using a Pall-Filtron Centramate open channel 100K MWCO membrane (2.0 sq. ft.). The filtration unit and membrane were first equilibrated with 1L of a solution of 10mM Tris-HCl, pH8.0. The buffer is circulated across the membrane using a pump and a sterile reservoir bottle. The DNA solution was added with the permeate channel fully open, and the solution circulated for approximately 30 min at 10 psi. The DNA solution was then ultrafiltered until concentrated to a volume of approximately 100 ml. The concentrated solution was then diafiltered using continuous diafiltration against a solution of 10mM Tris-HCl, pH 8, at 10 psi and permeate flow rate of 120 ml/min until the conductivity of the solution was decreased from an initial value of 35mS to less than 1mS (0.60) (buffer conductivity = 0.53mS). With the permeate valve closed, the retentate was then recirculated through the ultrafilter for 10min. The retentate was then collected, and the membrane washed with three 100 ml washes of 10mM Tris-HCl, pH 8.0.

[0073] The DNA and endotoxin concentrations of the diafiltered DNA solution and each of the washes was determined as above. The retentate and first wash were pooled, yielding 1.366g DNA at a concentration of 5.564mg/ml, and 90.35 EU/ml or 16.24 EU/mg DNA. This DNA solution was filtered through a Millipore Millipak 40 0.22µm filter, followed by filtration of an additional 25ml of final diafiltration buffer, and the two solutions pooled to yield the final product.

[0074] Yield of final plasmid DNA product from the final ultrafiltration was 80%. The final product was then aliquotted and stored at -20°C until use. The final product was determined to meet the following Quality Control specifications:

Color	clear to slightly cloudy
Endotoxin	< 100 EU/ml
Purity	> 95% by HPLC
DNA homogeneity	> 90% ccc (covalently closed circular)
RNA	< 2% by analytical HPLC
ssDNA	< 1% by analytical HPLC
Protein	< 0.1% by analytical HPLC. silver stain and SDS-PAGE
Genomic DNA	< 1% by analytical HPLC and Southern Blot
Conductivity	< 1 mS
pH	7-8.5

[0075] Sterility was assayed by day 21 tryptose broth culture showing no colonies. Identity was determined by re-

striction endonuclease digestion and analysis by agarose gel electrophoresis. Results of the analysis of p4119 are shown in Figure 5. HPLC analysis of the final product is shown in Figure 6.

[0076] It will be understood by those of skill in the art that the degree of purity achieved using the methods of the present invention will depend on the intended uses of the purified nucleic acid. Accordingly, the invention is intended to encompass embodiments whereby any degree of purity, including the degree of purity illustrated by the examples herein, can be achieved. The invention is therefore not limited in scope to the use of the methods of the invention in their most optimal embodiments or to produce nucleic acid of the maximum obtainable purity.

[0077] It will be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications and alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

Claims

1. A method for purifying a nucleic acid from a solution comprising the nucleic acid, the method comprising:

   a) filtering the solution through an ultrafiltration unit comprising a gel layer to provide a permeate solution and a retentate solution whereby the nucleic acid is retained in the retentate solution, and
   b) collecting the retentate solution, whereby a purified nucleic acid solution is obtained.

2. A method for filtering a solution comprising a nucleic acid, the method comprising:

   a) filtering the solution through an ultrafiltration unit comprising a gel layer to provide a permeate solution and a retentate solution whereby the nucleic acid is retained in the retentate solution, and
   b) collecting the retentate solution, whereby a filtered nucleic acid solution is obtained.

3. The method of Claim 1 or Claim 2, wherein the nucleic acid is DNA or RNA.

4. The method of Claim 3, wherein the DNA is plasmid DNA or viral DNA.

5. The method of Claim 3, wherein the RNA is viral RNA.

6. The method of Claim 4, wherein the DNA is plasmid DNA and the ultrafiltration unit comprises a membrane having a molecular weight cutoff of from about 50K to about 500K daltons.

7. A method for purifying plasmid DNA from a solution comprising the plasmid DNA, the method comprising the steps of:

   a) filtering the solution through an open-channel ultrafiltration unit comprising a membrane having a molecular weight cutoff in the range of from about 50K to about 500K daltons to provide a permeate solution and a retentate solution, whereby the plasmid DNA is retained in the retentate solution;
   b) collecting the retentate solution to provide a purified plasmid DNA solution.

8. A method for filtering a solution comprising a plasmid DNA, the method comprising the steps of:

   a) filtering the solution through an open-channel ultrafiltration unit comprising a membrane having a molecular weight cutoff in the range of from about 50K to about 500K daltons to provide a permeate solution and a retentate solution, whereby the plasmid DNA is retained in the retentate solution,
   b) collecting the retentate solution to provide a filtered DNA solution.

9. The method of Claim 7 or Claim 8, wherein the filtering is performed in the presence of a gel layer.

10. The method of any one of Claims 6 to 9, wherein the ultrafiltration membrane has a molecular weight cutoff of about 300K daltons.

11. The method of any one of Claims 7 to 10, further comprising diafiltering the solution to provide a volume exchange of from about 10 to 100 volume equivalents of the retentate solution.

12. The method of any one of Claims 1 to 6 or 9 to 11, wherein the gel layer is formed under pressure of from about 34.5 KPascal to about 206.8 KPascal (about 5 psi to about 30 psi).

13. The method of any one of Claims 1 to 12, further comprising concentrating the solution to provide a concentrated nucleic acid solution and diafiltering the concentrated nucleic acid solution with a diafiltration buffer.

14. The method of Claim 13, further comprising circulating the diafiltration buffer through the ultrafiltration unit after collecting the retentate solution, to provide a wash solution and combining the wash solution with the retentate solution.

15. The method of any one of the preceding Claims, further comprising the step of filtering the purified or filtered nucleic acid solution through a 0.2μm filter.

16. The method of any one of the preceding Claims, further comprising the step of applying the purified or filtered nucleic acid solution to a positively charged ion exchange chromatography resin wherein the nucleic acid is eluted from the ion exchange chromatography resin with a saline gradient to provide an eluted nucleic acid solution.

17. The method of Claim 16, further comprising the step of diafiltering the eluted nucleic acid solution.

18. The method of Claim 1, wherein the purified nucleic acid solution comprises at least about 90% pure nucleic acid or at least about 90% closed, circular plasmid DNA.

19. A method for preparing purified plasmid DNA from a solution comprising the nucleic acid, the method comprising the steps of:

a) circulating the solution through an open channel ultrafiltration unit comprising a membrane having a molecular weight cutoff in the range of from about 300K daltons to about 500K daltons under pressure of from about 5 psi (34.5 KPascal) to about 30 psi (206.8 KPascal) for sufficient time to allow a gel layer to form;
b) filtering the solution through the ultrafiltration unit comprising a gel layer to provide a retentate solution and a permeate solution whereby the plasmid DNA is retained in the retentate solution;
c) diafiltering the retentate solution with a diafiltration buffer;
d) collecting the retentate solution;
e) filtering the combined retentate solution through a 0.2μm filter to provide a substantially purified plasmid DNA solution;
f) applying the substantially purified plasmid DNA solution to a positively charged ion exchange chromatography resin wherein the plasmid DNA is eluted from the ion exchange chromatography resin with a saline gradient to provide an eluted plasmid DNA solution;

whereby a purified plasmid DNA solution is obtained.

20. The method of any one of Claims 7 to 19, wherein the solution comprising the plasmid DNA is obtained from the lysis of bacterial cells using a detergent-containing buffer and the substantial removal of cellular debris and proteins.

21. The method of any one of the preceding Claims, wherein the ultrafiltration unit is an open-channel device.

22. The method of Claim 21, wherein the ultrafiltration unit is a flat-plate device or a hollow fiber device, or a tangential flow device.

23. A method for purifying plasmid DNA from a mixture of cells, comprising the steps of:

a) disrupting the integrity of the cells comprising the mixture of cells in a detergent-containing buffer solution to provide a solubilized cell solution;
b) enzymatically digesting cellular RNA;
c) differentially precipitating cellular debris and proteins from nucleic acid in the solubilized cell solution to provide a supernatant solution comprising the nucleic acid;
d) removing the precipitated cellular debris and proteins from the supernatant solution to provide a substantially purified plasmid DNA solution;
e) further purifying the plasmid DNA by filtering the substantially purified plasmid DNA solution by tangential

flow ultrafiltration to provide a permeate solution and a retentate solution whereby the plasmid DNA is retained in the retentate solution;
f) collecting the retentate solution;

whereby a purified plasmid DNA composition is obtained.

24. A pharmaceutical composition comprising nucleic acid prepared according to the method of any one of the preceding Claims.

25. A pharmaceutical composition as claimed in Claim 24 wherein the nucleic acid is plasmid DNA.

26. A pharmaceutical composition according to Claim 25 comprising at least about 90% closed circular plasmid DNA.

27. A pharmaceutical composition according to Claim 25 or Claim 26 comprising:

less than about 100 endotoxin units per milligram plasmid DNA, or
less than about 2% RNA, or
less than about 1% single stranded DNA, or
less than about 0.1% protein, or
less than about 1% genomic DNA.

Patentansprüche

1. Verfahren zur Reinigung einer Nukleinsäure aus einer die Nukleinsäure umfassenden Lösung, wobei das Verfahren umfasst:

(a) Filtrieren der Lösung durch eine Ultrafiltrationseinheit, umfassend eine Gelschicht, um eine Permeat-Lösung und eine Retentat-Lösung bereitzustellen, wobei die Nukleinsäure in der Retentat-Lösung zurückgehalten wird, und
(b) Sammeln der Retentat-Lösung, wobei eine gereinigte Nukleinsäure-Lösung erhalten wird.

2. Verfahren zum Filtrieren einer eine Nukleinsäure umfassende Lösung, wobei das Verfahren umfasst:

(a) Filtrieren der Lösung durch eine Ultrafiltrationseinheit, umfassend eine Gelschicht, um eine Permat-Lösung und eine Retentat-Lösung bereitzustellen, wobei die Nukleinsäure in der Retentat-Lösung zurückgehalten wird, und
(b) Sammeln der Retentat-Lösung, wobei eine filtrierte Nukleinsäure-Lösung erhalten wird.

3. Verfahren nach Anspruch 1 oder Anspruch 2, worin die Nukleinsäure DNA oder RNA ist.

4. Verfahren nach Anspruch 3, worin die DNA Plasmid-DNA oder virale DNA ist.

5. Verfahren nach Anspruch 3, worin die RNA virale RNA ist.

6. Verfahren nach Anspruch 4, worin die DNA Plasmid-DNA ist und die Ultrafiltrationseinheit eine Membran mit einer Molekulargewichts-Abtrennung von etwa 50K bis etwa 500K Dalton umfasst.

7. Verfahren zur Reinigung von Plasmid-DNA aus einer die Plasmid-DNA umfassenden Lösung, wobei das Verfahren die Schritte umfasst:

(a) Filtrieren der Lösung durch eine offenkanalige Ultrafiltrationseinheit, umfassend eine Membran mit einer Molekulargewichts-Abtrennung im Bereich von etwa 50K bis etwa 500K Dalton, um eine Permat-Lösung und eine Retentat-Lösung bereitzustellen, wobei die Nukleinsäure in der Retentat-Lösung zurückgehalten wird, und
(b) Sammeln der Retentat-Lösung, um eine gereinigte Plasmid-DNA-Lösung bereitzustellen.

8. Verfahren zum Filtrieren einer Lösung, umfassend eine Plasmid-DNA, wobei das Verfahren die Schritte umfasst:

(a) Filtrieren der Lösung durch eine offenkanalige Ultrafiltrationseinheit, umfassend eine Membran mit einer Molekulargewichts-Abtrennung im Bereich von etwa 50K bis etwa 500K Dalton, um eine Permat-Lösung und eine Retentat-Lösung bereitzustellen, wobei die Plasmid-DNA in der Retentat-Lösung zurückgehalten wird, und
(b) Sammeln der Retentat-Lösung, um eine filtrierte DNA-Lösung bereitzustellen.

9. Verfahren nach Anspruch 7 oder Anspruch 8, worin die Filtration in Gegenwart einer Gelschicht durchgeführt wird.

10. Verfahren nach einem der Ansprüche 6 bis 9, worin die Ultrafiltrationsmembran eine Molekulargewichts-Abtrennung von etwa 300K Dalton besitzt.

11. Verfahren nach einem der Ansprüche 7 bis 10, weiter umfassend Diafiltrieren der Lösung, um einen Volumenaustausch von etwa 10 bis 100 Volumen-Äquivalenten der Retentat-Lösung bereitzustellen.

12. Verfahren nach einem der Ansprüche 1 bis 6 oder 9 bis 11, worin die Gelschicht unter einem Druck von etwa 34,5 KPascal bis etwa 206,8 KPascal (etwa 5 psi bis etwa 30 psi) gebildet wird.

13. Verfahren nach einem der Ansprüche 1 bis 12, weiter umfassend Aufkonzentrieren der Lösung, um eine konzentrierte Nukleinsäure-Lösung bereitzustellen, und Diafiltrieren der konzentrierten Nukleinsäure-Lösung mit einem Diafiltrationspuffer.

14. Verfahren nach Anspruch 13, weiter umfassend Zirkulieren des Diafiltrationspuffers durch die Ultrafiltrationseinheit nach dem Sammeln der Retentat-Lösung, um eine Waschlösung bereitzustellen, und Vereinigen der Waschlösung mit der Retentat-Lösung.

15. Verfahren nach einem der vorhergehenden Ansprüche, weiter umfassend den Schritt Filtrieren der gereinigten oder filtrierten Nukleinsäure-Lösung durch einen 0,2 μm Filter.

16. Verfahren nach einem der vorhergehenden Ansprüche, weiter umfassend den Schritt Auftragen der gereinigten oder filtrierten Nukleinsäure-Lösung auf ein positiv geladenes Ionenaustausch-Chromatographieharz, wobei die Nukleinsäure mit einem Salzgradienten von dem Ionenaustausch-Chromatographieharz eluiert wird, um eine eluierte Nukleinsäure-Lösung bereitzustellen.

17. Verfahren nach Anspruch 16, weiter umfassend den Schritt Diafiltrieren der eluierten Nukleinsäure-Lösung.

18. Verfahren nach Anspruch 1, worin die gereinigte Nukleinsäure-Lösung mindestens etwa 90% reine Nukleinsäure oder mindestens etwa 90% geschlossene, zirkuläre Plasmid-DNA umfasst.

19. Verfahren zur Herstellung einer gereinigten Plasmid-DNA aus einer die Nukleinsäure umfassenden Lösung, wobei das Verfahren die Schritte umfasst:

a) Zirkulieren der Lösung durch eine offenkanalige Ultrafiltrationseinheit, umfassend eine Membran mit einer Molekulargewichts-Abtrennung im Bereich von etwa 300K Dalton bis etwa 500K Dalton, unter einem Druck von etwa 5 psi (34,5 KPascal) bis etwa 30 psi (206,8 KPascal) für eine Zeit, die ausreichend ist, damit eine Gelschicht gebildet werden kann;
b) Filtrieren der Lösung durch eine Ultrafiltrationseinheit, umfassend eine Gelschicht, um eine Permat-Lösung und eine Retentat-Lösung bereitzustellen, wobei die Plasmid-DNA in der Retentat-Lösung zurückgehalten wird;
c) Diafiltrieren der Retentat-Lösung mit einem Diafiltrationspuffer;
d) Sammeln der Retentat-Lösung;
e) Filtrieren der vereinigten Retentat-Lösung durch einen 0,2 μm Filter, um eine im Wesentlichen gereinigte Plasmid-DNA-Lösung bereitzustellen;
f) Auftragen der im Wesentlichen gereinigten Plasmid-DNA-Lösung auf ein positiv geladenes Ionenaustausch-Chromatographieharz, wobei die Plasmid-DNA mit einem Salzgradienten von dem Ionenaustausch-Chromatographieharz eluiert wird, um eine eluierte Plasmid-DNA-Lösung bereitzustellen;

wobei eine gereinigte Plasmid-DNA-Lösung erhalten wird.

20. Verfahren nach einem der Ansprüche 7 bis 19, worin die Lösung, umfassend die Plasmid-DNA, aus der Lyse bakterieller Zellen unter Verwendung eines detergenzienhaltigen Puffers und der wesentlichen Entfernung von Zelltrümmern und Proteinen erhalten wird.

21. Verfahren nach einem der vorhergehenden Ansprüche, worin die Ultrafiltrationseinheit eine offenkanalige Vorrichtung ist.

22. Verfahren nach Anspruch 21, worin die Ultrafiltrationseinheit eine Vorrichtung mit einer flachen Platte oder eine Hohlfaser-Vorrichtung oder eine Tangentialfluss-Vorrichtung ist.

23. Verfahren zur Reinigung von Plasmid-DNA aus einem Gemisch von Zellen, umfassend die Schritte:

a) Zerstören der Integrität der Zellen, umfassend das Gemisch aus Zellen in einer detergenzienhaltigen Pufferlösung, um eine solubilisierte Zell-Lösung bereitzustellen;
b) enzymatisches Verdauen zellulärer RNA;
c) differenzielles Abscheiden von Zelltrümmern und Proteinen von Nukleinsäure in der solubilisierten Zell-Lösung, um eine die Nukleinsäure umfassende Überstandslösung bereitzustellen;
d) Entfernen der präzipitierten Zelltrümmer und Proteine aus der Überstandslösung, um eine im Wesentlichen gereinigte Plasmid-DNA-Lösung bereitzustellen;
e) weiteres Reinigen der Plasmid-DNA durch Filtrieren der im Wesentlichen gereinigten Plasmid-DNA-Lösung durch eine Tangentialfluss-Ultrafiltration, um eine Permat-Lösung und eine Retentat-Lösung bereitzustellen, wobei die Plasmid-DNA in der Retentat-Lösung zurückgehalten wird;
f) Sammeln der Retentat-Lösung;

wobei eine gereinigte Plasmid-DNA-Zusammensetzung erhalten wird.

24. Pharmazeutische Zusammensetzung, umfassend eine Nukleinsäure, hergestellt durch das Verfahren nach einem der vorhergehenden Ansprüche.

25. Pharmazeutische Zusammensetzung nach Anspruch 24, worin die Nukleinsäure Plasmid-DNA ist.

26. Pharmazeutische Zusammensetzung nach Anspruch 25, umfassend mindestens etwa 90% geschlossene zirkuläre Plasmid-DNA.

27. Pharmazeutische Zusammensetzung nach Anspruch 25 oder Anspruch 26, umfassend:

weniger als etwa 100 Endotoxin-Einheiten pro Milligramm Plasmid-DNA, oder weniger als etwa 2% RNA, oder
weniger als etwa 1 % Einzelstrang-DNA, oder
weniger als etwa 0,1 % Protein, oder
weniger als etwa 1% genomische DNA.

Revendications

1. Procédé de purification d'un acide nucléique à partir d'une solution contenant l'acide nucléique, ledit procédé comprenant les étapes consistant à :

a) filtrer de la solution au travers d'une unité d'ultrafiltration comprenant une couche d'un gel, de façon à obtenir une solution de perméat et une solution de rétentat, ce par quoi l'acide nucléique est retenu dans la solution de rétentat, et

b) récupérer la solution de rétentat, ce par quoi une solution d'acide nucléique purifié est obtenue.

2. Procédé de filtration d'une solution contenant un acide nucléique, ledit procédé comprenant les étapes consistant à :

a) filtrer la solution au travers d'une unité d'ultrafiltration comprenant une couche d'un gel, de façon à obtenir une solution de perméat et une solution de rétentat, ce par quoi l'acide nucléique est retenu dans la solution

de rétentat, et

b) récupérer la solution de rétentat, une solution d'acide nucléique purifié étant ainsi obtenue.

3. Procédé selon la revendication 1 ou la revendication 2, où l'acide nucléique est de l'ADN ou de l'ARN.

4. Procédé selon la revendication 3, où l'ADN est de l'ADN plasmidique ou de l'ADN viral.

5. Procédé selon la revendication 3, où l'ARN est de l'ARN viral.

6. Procédé selon la revendication 4, où l'ADN est de l'ADN plasmidique et l'unité d'ultracentrifugation comprend une membrane ayant un poids moléculaire de coupure allant d'environ 50 à environ 500 kdaltons.

7. Procédé de purification d'un ADN plasmidique à partir d'une solution contenant l'ADN plasmidique, ledit procédé comprenant les étapes consistant à

a) filtrer la solution au travers d'une unité d'ultrafiltration à voie principale ouverte comprenant une membrane d'un poids moléculaire de coupure compris dans une gamme d'environ 50 à environ 500 kdaltons de façon à obtenir une solution de perméat et une solution de rétentat, ce par quoi l'ADN plasmidique est retenu dans la solution de rétentat, et

b) récupérer la solution de rétentat, ce par quoi on obtient une solution d'ADN plasmidique purifié.

8. Procédé de filtration d'une solution contenant un ADN plasmidique, ledit procédé comprenant les étapes consistant à:

a) filtrer la solution au travers d'une unité d'ultrafiltration à voie principale ouverte comprenant une membrane d'un poids moléculaire de coupure compris dans une gamme d'environ 50 à environ 500 kdaltons afin d'obtenir une solution de perméat et une solution de rétentat, ce par quoi l'ADN plasmidique est retenu dans la solution de rétentat et

b) récupérer la solution de rétentat, ce par quoi on obtient une solution d'ADN filtré.

9. Procédé selon la revendication 7 ou la revendication 8, dans lequel la filtration est effectuée en présence d'une couche d'un gel.

10. Procédé selon l'une quelconque des revendications 6 à 9, dans lequel la membrane d'ultrafiltration a un poids moléculaire de coupure d'environ 300 kdaltons

11. Procédé selon l'une quelconque des revendications 7 à 10, comprenant en une étape supplémentaire de diafiltration de la solution de façon à obtenir un volume d'échange d'environ 10 à 100 volumes équivalents de la solution de rétentat.

12. Procédé selon l'une quelconque des revendications 1 à 6 ou 9 à 11, dans lequel la couche de gel est formée sous une pression d'environ 34,5 kPascal à environ 206,8 kPascal (d'environ 5 psi à environ 30 psi).

13. Procédé selon l'une quelconque des revendications 1 à 12, comprenant en outre une étape de concentration de la solution de façon à obtenir une solution d'acide nucléique concentrée et la diafiltration de la solution d'acide nucléique concentrée avec un tampon de diafiltration

14. Procédé selon la revendication 13, comprenant en outre une étape de circulation du tampon de diafiltration au travers de l'unité d'ultrafiltration après avoir recueilli la solution de rétentat, de façon à obtenir une solution de lavage et la combinaison de la solution de lavage avec la solution de rétentat.

15. Procédé selon l'une quelconque des revendications précédentes, comprenant en outre une étape de filtration de la solution d'acide nucléique purifié ou filtré sur un filtre dont les pores ont une taille de 0,2 µm.

16. Procédé selon l'une quelconque des revendications précédentes, comprenant en outre une étape consistant à

appliquer la solution d'acide nucléique purifiée ou filtrée sur une résine de chromatographie par échange d'ions chargée positivement, dans lequel l'acide nucléique est élué de la résine de chromatographie par échange d'ions avec un gradient de sel de façon à obtenir une solution d'acide nucléique élué.

17. Procédé selon la revendication 16 comprenant en outre une étape consistant à réaliser une diafiltration de la solution d'acide nucléique élué.

18. Procédé selon la revendication 1, dans lequel la solution d'acide nucléique purifié comprend au moins environ 90% d'acide nucléique pur, ou au moins 90% d'ADN plasmidique circulaire, fermé.

19. Procédé de préparation d'un ADN plasmidique purifié à partir d'une solution comprenant l'acide nucléique, ledit procédé comprenant les étapes consistant à :

a) faire circuler la solution au travers d'une unité d'ultrafiltration à voie principale ouverte comprenant une membrane d'un poids moléculaire d'exclusion compris dans une gamme d'environ 300 à environ 500 kdaltons sous une pression d'environ 34,5 kPascal à environ 206,8 kPascal (environ 5 psi à environ 30 psi) pendant une durée suffisante pour qu'il se forme un gel ;

b) filtrer la solution au travers d'une unité d'ultrafiltration comprenant une couche de gel afin d'obtenir une solution de perméat et une solution de rétentat, l'ADN plasmidique étant retenu dans la solution de rétentat ;

c) réaliser une diafiltration de la solution de rétentat avec un tampon de diafiltration ;

d) récupérer la solution de rétentat ;

e) filtrer la solution de rétentat combinée sur un filtre de 0,2µm pour obtenir une solution d'ADN plasmidique essentiellement purifié ;

f) déposer la solution d'ADN plasmidique pratiquement purifié sur une résine de chromatographie par échange d'ions chargée positivement où l'ADN plasmidique est élué de la résine de chromatographie par échange d'ions avec un gradient de sel de façon à obtenir une solution d'ADN plasmidique élue ;

ce par quoi on obtient une solution d'ADN plasmidique purifié.

20. Procédé selon l'une quelconque des revendications 7 à 19, dans lequel la solution contenant l'ADN plasmidique est obtenu en lysant des cellules bactériennes à l'aide d'un tampon contenant un détergent et en éliminant essentiellement tous les débris cellulaires et les protéines.

21. Procédé selon l'une quelconque des revendications précédentes, dans lequel le dispositif d'ultrafiltration est un dispositif à voie principale ouverte.

22. Procédé selon la revendication 21, dans lequel l'unité d'ultrafiltration est un dispositif à plateau plat ou un dispositif à fibres creuses, ou un dispositif à écoulement tangentiel.

23. Procédé de purification d'un ADN plasmidique à partir d'un mélange de cellules, comprenant les étapes consistant à :

a) effectuer une rupture de l'intégrité des cellules, cette étape comprenant un mélange des cellules avec une solution tampon contenant un détergent de façon à obtenir une solution de cellules solubilisées ;

b) digérer l'ARN cellulaire par traitement enzymatique ;

c) précipiter différentiellement les débris cellulaires et les protéines des acides nucléiques dans la solution de cellules solubilisées de manière à obtenir une solution de surnageant contenant l'acide nucléique ;

d) éliminer les débris cellulaires et les protéines précipités de la solution de surnageant de manière à obtenir une solution d'ADN plasmidique essentiellement purifié ;

e) purifier encore l'ADN plasmidique en filtrant la solution d'ADN plasmidique pratiquement purifié par ultrafiltration à écoulement tangentiel de manière à obtenir une solution de perméat et une solution de rétentat, ce par quoi l'ADN plasmidique est retenu dans la solution de rétentat ;

f) récupérer la solution de rétentat ;

ce par quoi on obtient une composition d'ADN plasmidique purifiée.

24. Composition pharmaceutique comprenant un acide nucléique préparé selon le procédé de l'une quelconque des revendications précédentes.

25. Composition pharmaceutique selon la revendication 24 dans laquelle l'acide nucléique est un ADN plasmidique.

26. Composition pharmaceutique selon la revendication 25 comprenant au moins environ 90% d'ADN plasmidique circulaire fermé.

27. Composition pharmaceutique selon la revendication 25 ou la revendication 26 comprenant :

moins d'environ 100 unités d'endotoxine par milligramme d'ADN plasmidique, ou
moins d'environ 2% d'ARN, ou
moins d'environ 1% d'ADN simple brin, ou
moins d'environ 0,1% de protéines, ou
moins d'environ1% d'ADN génomique.

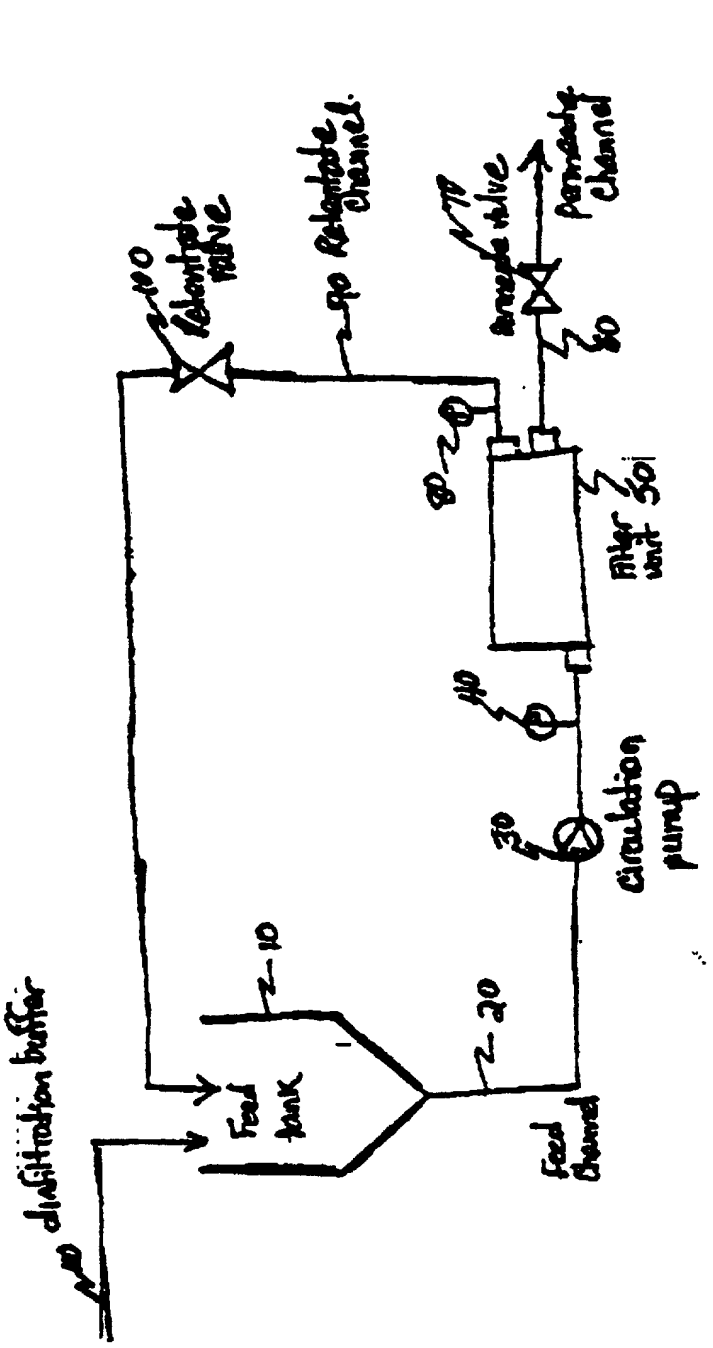
diafiltration buffer
Feed tank
10
20
Feed channel
30
circulation pump
40
filter unit
50
60
Permeate channel
70
Retentate valve
80
90
Retentate channel

Figure 1

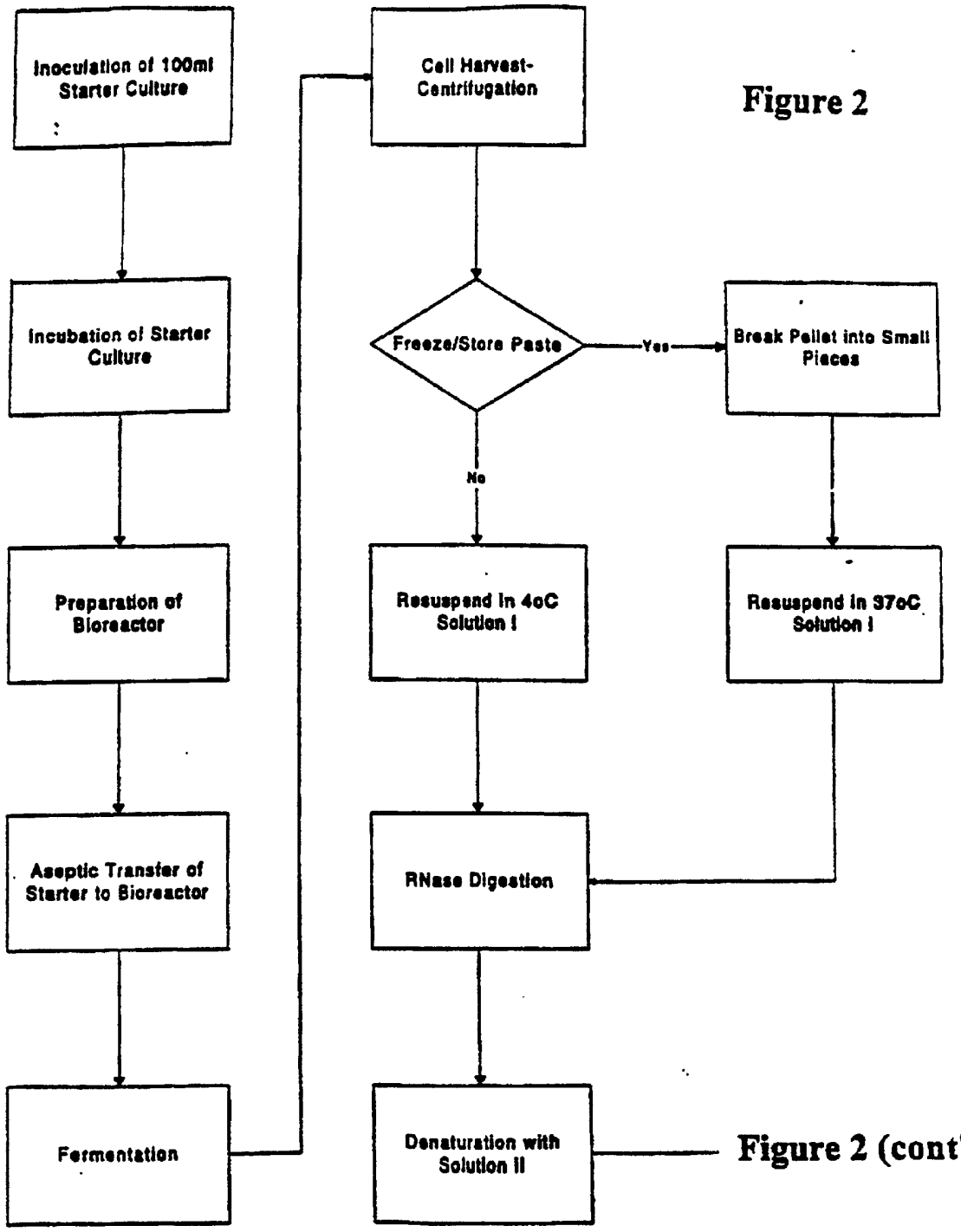

Figure 2

Figure 2 (cont'd.)

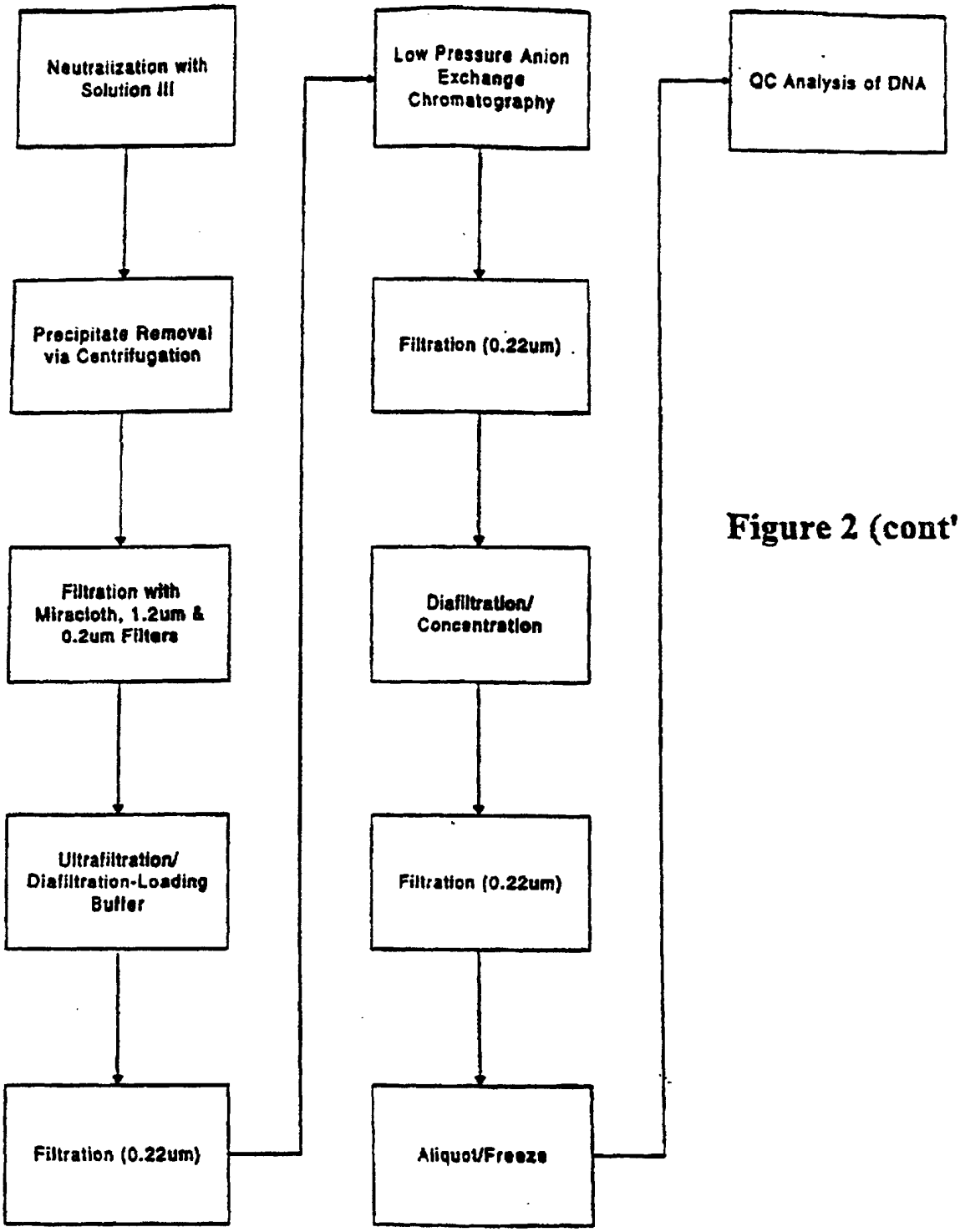

Figure 2 (cont'd.)

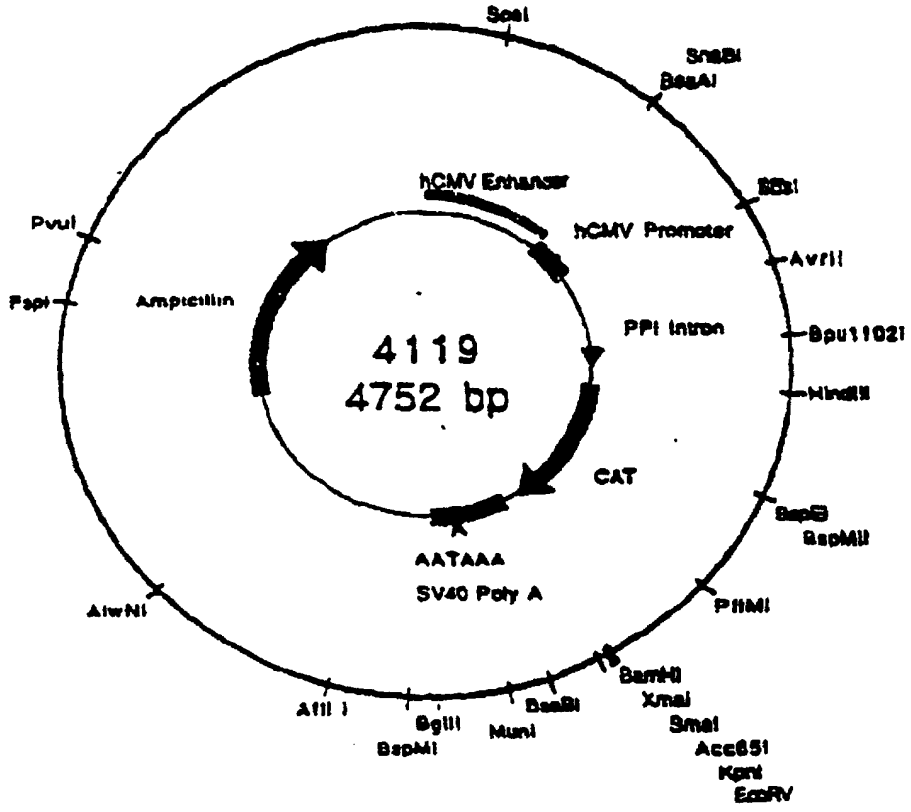
SseI
SnaBI
BssAI
hCMV Enhancer
hCMV Promoter
BSsI
Pvul
AvrII
Fspl
Ampicillin
PPI Intron
Bpu1102I
4119
4752 bp
HindIII
CAT
BspEI
BspMII
AATAAA
SV40 Poly A
AlwNI
PflMI
BamHI
Xmai
SmaI
Acc65I
KpnI
EcoRV
AflII
BglII
BspMI
BssBI
MunI

Figure 3

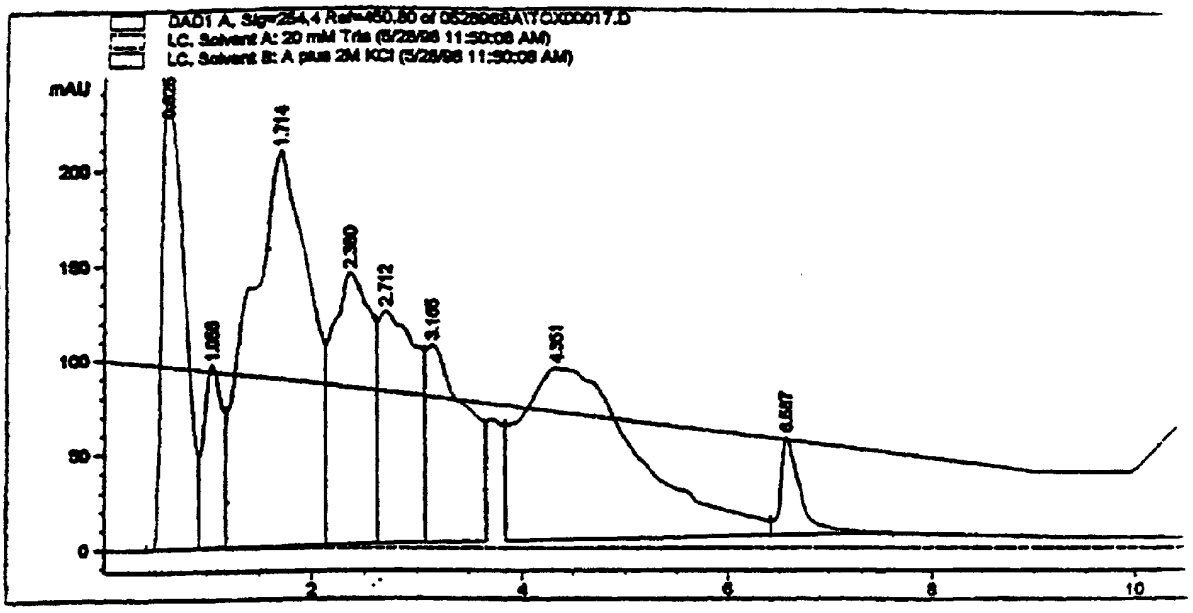
LC, Solvent A: 20 mM Tris (5/28/98 11:50:08 AM)
LC, Solvent B: A plus 2M KCl (5/28/98 11:50:08 AM)
mAU
200
150
100
50
0
1.714
2.380
2.712
3.165
4.351
6.567
2
4
6
8
10

Figure 4A

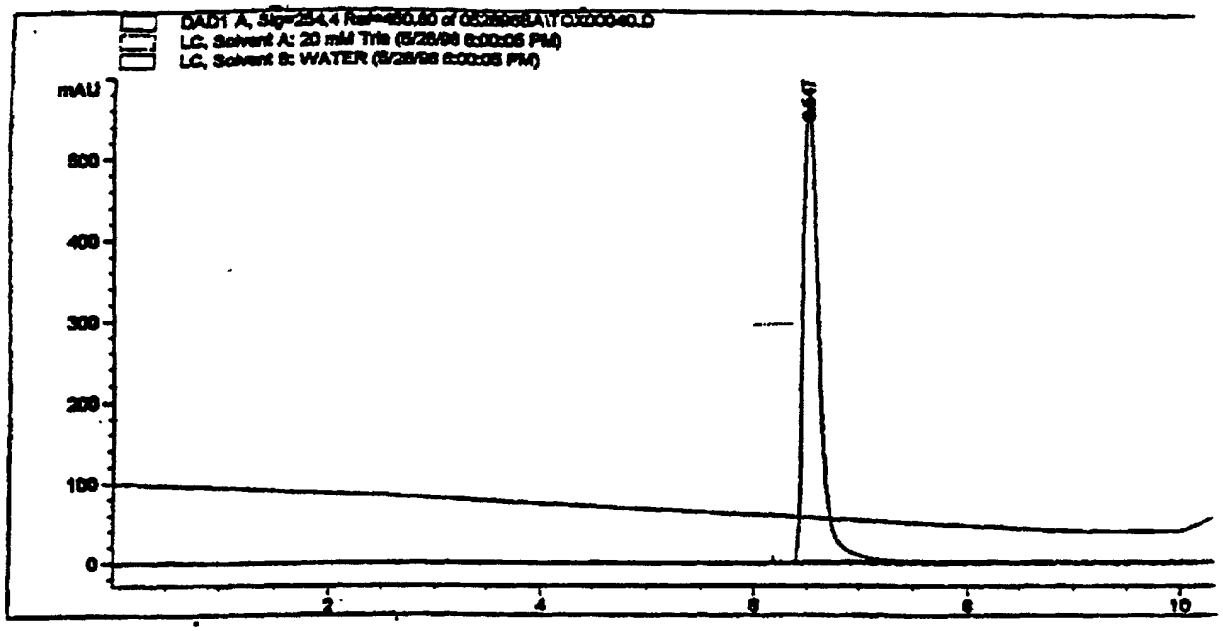
LC, Solvent A: 20 mM Tris
LC, Solvent B: WATER
mAU
800
400
300
200
100
0
2
4
8
6
10

Figure 4B

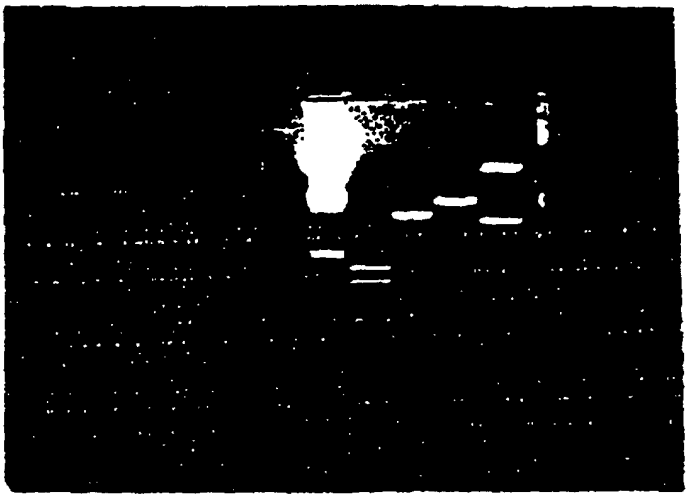

Figure 5

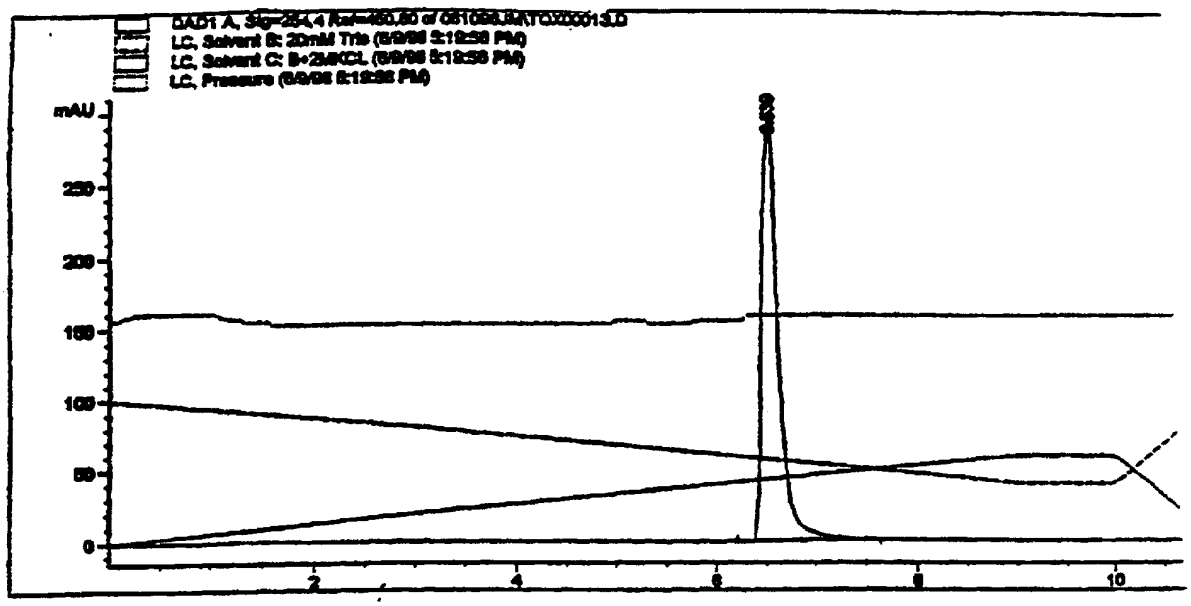

Figure 6